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Interlaboratory Comparison of Continuous Flow, Early Life Stage Testing with Fathead Minnows

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16. ABSTRACT

Six laboratories conducted toxicity experiments according to a supplied protocol. Also supplied were the chemicals to be tested (acenaphthene and isophorone). Test organisms were fathead minnow (Pimephales promelas) embryos which were raised until 28 days post hatch. All fish were weighed and compared with the controls by each investigator and all analytical work and other necessities were provided by the various participants. Results ranged between 0.049 mg/1 and 0.42 mg/1 for the low solubility acenaphthene and between 1.35 mg/1 and 45.4 mg/1 for a more soluble isophorone. The isophorone results were strongly correlated inversely to growth of the controls which varied between 0.969 gr and 0.018 gr for a high and low, respectively.

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Interlaboratory Comparison of Continuous Flow, Early Life Stage Testing with Fathead Minnows

A. E. Lemke

Toxicity testing with various organisms and various life stages is an important part of environmental protection strategy. The fathead minnow (Pimephales promelas) is a small cyprinid, well suited to laboratory conditions. Tests have been conducted on various materials using acute (96 hr), sub-chronic (embryo, larval, juvenile, 30 day), and full life history techniques (6-8 months).

Toxicity testing data summarized by McKim (1977) indicates that the early life stages, particularly at the time the young fish begin to feed after absorbing their yolk sac, are the most sensitive.

A set of guidelines (attached) for the fathead minnow embryo larval test was prepared by the research group at the Environmental Research Laboratory-Duluth of the U.S. EPA. Seven laboratories participated in the evaluation of these guidelines. The participating laboratories will be designated by the following acronynms: (ANSP, ERCO, MBL, JEI, ABI, WFTS, ERL-D). Laboratory names, addresses and principal investigator are listed in Appendix 1.

General Procedures

A request for proposals was initiated through the EPA contracting office. This proposal contained the guidelines, a rating scheme for evaluation, and a scope of work. The scope of work requested that two chemicals, acenaphthene and isophorone, be tested twice each in tests beginning with less than 24 hr old embryos and terminating 28 days after hatching. The guidelines called for the principal reporting parameter to be the nonlethal effect of growth different from the controls with 96 hr LC50

and 30 day LC50 also to be reported if developed as part of the growth differential testing.

The highest concentration not different from the control and below which all concentrations were not different from the control was designated as the lower chronic endpoint. Conversely, the lowest concentration which was different from the control and above which all concentrations were different from the control was called the upper chronic endpoint. These were compared. Other parameters compared were number of test concentrations, toxicant preparation solvents, number of embryos initially, chamber size, embryo cup construction, water supply, photoperiod, temperature, dissolved oxygen, flow rate, culture techniques of stock culture, acquisition and handling of embryos initially, control conditions, feeding (timing, kind, amount), tank cleaning, termination techniques, and chemistry of both compounds. All comparisons were made in relation to the guidelines as written. If no specific suggestions were made, the individual labs compared with each other and ERL-D experience.

Finally, suggestions for improving the guidelines were requested.

Procedure Adherence by Participating Laboratories

The guidelines recommended that the test water should allow rearing of fish and other aquatic organisms. Water quality parameters called for included, temperature 25 $\pm$ 1° C, pH 7.1-8.5, dissolved oxygen between 75 and 100 of saturation, and flow rate sufficient to prevent loss of toxicant concentration in contact with the test organisms. These parameters have been found to provide a suitable base for good survival and growth of fathead minnow embryos and larva. Other recommendations are found in the guidelines (attached). All laboratories followed water quality procedures well within

the guidelines. Hardness varied from a high of 390-310 mg/l $CaCO_3$ (ERCO) to a constant low of 47 mg/l $\pm$ 1.5 mg (ERL-D) and a variable low between 27-70 (WFTS). All of the laboratories used the test water for culture purposes also.

pH levels reported by all but one laboratory were well within the satisfactory range of 7.1 - 8.5. MBL did not report pH.

Temperatures in the tests were well controlled with all laboratories reporting a mean of 25°C $\pm$ 1°C as recommended by the guidelines.

Four of the six laboratories used well water as dilution water (ERCO, MBL, JEI, API, and WFTS). One laboratory reported using unfiltered water from a pristine stream, the watershed of which is controlled by the laboratory (ANSP). The other laboratory used sand filtered Lake Superior water obtained by pipeline offshore (ERL-D).

The other water parameter asked for in the guidelines was dissolved oxygen. All laboratories reported dissolved oxygen levels of +70%. WFTS reported concentration 6.2 - 8.4 mg/D.O./l which converts to 74 - 98% saturation at the reported test temperature. One laboratory (MBL) did not follow the guidelines. Instead, they maintained the dissolved oxygen by aeration of the test tanks.

Guideline test conditions concerned with operational procedures included starting numbers of embryos, sorting, water turnovers, test chamber size (Table 1), use of solvents to disperse the test materials, and number of test concentrations.

Embryo Handling

The guidelines recommended 60 embryos or more per concentration initially. ABI used 40 per concentration in each test and ERL-D used 40 in one test and 60 or more in the other three. The sorting or reduction in

numbers of organisms before transfer into the rearing tanks was not recommended. ANSP and WFTS reduced the final number of larva to 40 for rearing, all others transferred all hatched animals. All participants used duplicate chambers and five concentrations and a control. They replicated by space and/or time, giving 4 separate tests as requested in the scope of work. Exact turnover rates were not specified in the guidelines other than that they should be sufficient to keep the toxicant concentration stable and dissolved oxygen at +75% saturation. Turnover rates from 8 to 88 times in 24 hrs. were reported. MBL did not report turnover rate.

All laboratories used diethylformamide as a solvent for the acenapthene which is only sparingly soluble in water. MBL and JEI reported difficulty with the solvent. Isophorone was added directly to the test water by all reporting units except JEI which used a solvent.

Chemical Methods

Chemical analyses of the test concentrations were accomplished by the following methods.

Analytical Chemistry Methods

Laboratory	Acenapthene	Isophorone
ANSP	Gas liquid chromatograph	UV spectroscopy
ERCO	Fluorometric	Gas chromatograph
MBL	Flourometric	Gas chromatograph
JEI	Gas chromatograph	Gas chromatograph
ABI	Gas chromatograph	Gas chromatograph
WFTS	High pressure liquid chromatograph	Gas chromatograph
ERL-D	Flourometric	Gas chromatograph

Analytical problems were reported by MBL and JEI. They both reported difficulty with flocculent material associated with the dimethyl foramide used as the solvent for acenapthene. MBL examined some of their flocculent material and found large numbers of ciliates associated with the floc.

Table 1

Chamber Size (in inches) and Egg Cup Condition

	L	W	H	Agitation of Embryo Cups
ANSP	40	20	26	No
ERCO	18	6	8	Yes
MBL	34.5	15	14.5	Yes
JEI	25	16	17	Stirred water
ABI	24	19	19.9	Yes (siphon)
WFTS	9	9	10.5	Yes (screen tray)
ERL-D	6	16	18	Yes

Table 1 shows the chamber sizes utilized by the participants for fry rearing. This range of chamber sizes is as large or larger than suggested in the guidelines. The guidelines called for making sure that the eggs (embryos) were exposed to the toxicant and sufficient oxygenated test water. Gentle agitation in a reciprocating system was suggested but not mandatory. Only ANSP did not provide any special consideration for insuring water flow around the embryos.

Feeding of Fry

All laboratories fed brine shrimp (Artemia salina) nauplii at least twice a day throughout the test except on weekends when they were fed once by most participants (ANSP fed twice daily on weekends). ERL-D supplemented the

brine shrimp with fine particle trout chow and ANSP reported a large amount of natural food, i.e., copepods, etc., in their unfiltered stream water.

No mention was made by any laboratories which day in relation to the weekend the tests were started with the exception of ERL-D. Their report expressed a belief that a shortened early feeding schedule (larva emerged on weekend) on one test accounted for less, but still satisfactory growth than the other three tests.

Test Results

The effect/no effect levels as reported by the participating laboratories are presented in Table 2. The acenaphthene, no effect means range from a low of .049 mg/l to a high of .42 mg/l or approximately a 10-fold difference. Isophorone no effect means have a greater range 1.35 mg/l to 45.4 mg/l, a 40-fold difference.

Growth variations as measured by reported values of control fish weights (Table 2) vary from a high of .969 gr for ANSP to a low of .0018 gr for MBL, nearly 3 orders of magnitude (>50 times). That indicates problems with the feeding regime.

An anomaly in the data set is the difference in ranges between weight, acenaphthene endpoints, and isophorone endpoints. To resolve the problem, regression analyses were conducted with each of the sets regressed against the others. No significant correlation was found with the acenapthene data versus either of the other sets. The weight versus the isophorone data resulted in an R^2 of .87 in the exponential least squares regression of the form $Y = Ae^{BX}$ where A = 24.66 and B = -3.087. Thus as the weight goes up, differences from the control are revealed at lower concentrations. This by itself is logical but the acenaphthene data does not show this effect.

Table 2

Interlaboratory Comparison Test Results

Participating Organization	Control Growth				Test Results No Effect Level			
	Ace 1	Ace 2	Iso 1	Iso 2	Ace 1	Ace 2	Iso 1	Iso 2
ANSP	.953 .948s	.969 .965s	.957	.969	.075-.18 (.16)	.18-.37 (.26)	.65-2.8 (1.35)	.65-2.8 (1.35)
ERCO	.0056	.0057	.0061	.0055	.064-.098 (.079)	.091-.139 (.112)	14.8-24.5 (19.0)	11.8-53.9 (25.2)
MBL	.0030 .0052s	NR .011s	.0095	.0018	.034-.071 (.049)	.033-.088 (.054)	31.5-65.4 (45.4)	29.3-56.1 (40.5)
JEI	.095	.012			-	-	-	-
ABI	.083 .085s	.091 .095s	.045	.088	.11-.24 (.16)	.12-.27 (.18)	9.2-20.0 (13.6)	19.9-40.6 (28.4)
WFTS	.031 .037s	.023	.026	-	.33-.50 (.41)	.35-.51 (.42)	11-19 (14.5)	-
ERL-D	.186 .218s	.122 .180	.141	.202	.133-.265 (.188)	.146-.285 (.204)	14.5-27.6 (20.0)	4.15-8.78 (6.0)

All weights are in mg and are mean weight of surviving control fish.

All participants met the criteria for control survival of 80% or better.

Result numbers in mg/l analyzed.

s is solvent control weights

(n) are geometric means

Discussion and Resolution of Procedural Problems

The difficulties encountered by participants in conducting the bioassays can be categorized in three ways: handling problems with the solvents and the chemicals, difficulty with the frequency of determinations to be taken, and test organism problems, especially lack of growth.

These problems are universal in bioassay work. Twenty-five years of experience by the author have shown all of these to be problems encountered by experimenters who are trying to follow a slightly different system than their usual routine. Experience with similar procedures reduces the problems but they are not eliminated. Discussion with two ERL-D testing crews (personal communication, Duane Benoit, Gary Phipps) indicated that chemical handling is a problem for even the most experienced testing units. Solubility, volatility, and cross-contamination between tests are always a problem. Each requires different resolution and, although they are not an actual part of the bioassay procedure, they do contribute to about one-third of the failures, especially as the types of compounds tested change.

Some work at our laboratory in this area of structure activity has shown that low water solubilities can cause unexpected results (Veith, personal communication). Three of the laboratories reported some solubility problems with acenapthene, and two used saturated solutions as the highest concentration. Isophorone did not cause this problem. The solubilities in water are 3.42 mg/l and 12,000 mg/l as acenapthene and isophorone, respectively (Callahan et al., 1979). The low solubility of acenaphthene supports the contention that the results of the acenaphthene tests are not affected by the differences in growth as were the isophorone tests.

The second problem area was reported by all contract laboratories. It concerned the actual number of analyses, type of analyses, and statistical

evaluations to be made during the chemical and biological data gathering. All of the laboratories preferred sets of standard forms for daily use and for compilation at the end of the test set. The contract laboratories also reported that they could do more accurate pricing if such a set of forms were included. Contract laboratories said a further benefit of a form set is that it would serve as a check list.

The other reported problem is the difficulty found in actually rearing and growing the test animals. Only three of the seven participating laboratories had satisfactory growth compared to that obtained by some of the guideline writers (Benoit et al., 1982; Jarvinen and Tanner, 1982). It is the opinion of the participants that the feeding regime suggested in the guideline is not specific enough, and that perhaps other foods should be tried. Suggestions include the use of protozoa cultures, prepared aquarium food, natural food from a clean water source, and special kinds of brine shrimp nauplii since apparently they are not all the same size depending on the source. Also considered to be a problem was the timing of the feeding during the first several days after the young fatheads absorb their yolk sac and begin to feed. ERL-D reported 44% more growth when the test animals hatched during the week rather than during the weekend. (The guidelines allow a reduced feeding schedule on the weekend.)

Resolution of the request for a set of data gathering forms is easy. Experience with four interlaboratory comparison test sets indicate that such forms should be added to the guidelines.

The other two problems, i.e., chemical handling and organism handling and feeding, are much less amenable to resolution.

Chemical handling revolves around system changes required for various chemicals. Metals and water soluble organics have entirely different

problems than either volatile organics or slightly water soluble materials. Experience with the type of chemical group in question is the best solution. Unfortunately, experience cannot be written into a set of protocols. The best generalization to alleviate the problem is to suggest that groups of chemicals be tested which have similar physical and toxicity characteristics. Materials like acetone and ethyl alcohol require different chemical handling than endrin and PCBs.

Liquids, solids, and biodegradable test materials require different systems. A contract group conducting a large number of flow through 96 hr acute bioassays with juvenile fathead minnows has developed four types of test systems (personal communication, Michael Knuth). Chemicals are assigned to the various systems on the basis of the parameters previously enumerated. The guidelines cover this area with the statement "suitable distribution devices to maintain test concentrations". Perhaps separate guidelines sections should be written for operation of the test systems based on the physical and chemical parameters of the test materials.

The wide variation in growth of the fish during the 30 day test period indicates that the food supply and/or food presentation is a problem in completing a satisfactory test based on the guidelines. Several reports, Beck et al. (1980), Johns et al. (1980), indicate that various brine shrimp are not alike in suitability as a food source for young fish. The experience of the author and various test groups at ERL-D also indicate that the age of the nauplii used, i.e., newly hatched versus late first instar also can affect the suitability as food for first feeding fathead minnows. This is perhaps caused by size or mobility differences as newly feeding fish are very selective (Siefert, 1972). Availability, and tank distribution also are factors based on observation. This is emphasized by the report of one

participant (ANSP) which had fish growth four times above the next two laboratories and ten to seven hundred times that reported by certain other participants. The natural food in that laboratory water supply, available continuously, was noted as the reason for this growth. Unfortunately, few laboratories have water with a good natural food supply and those that do have food that varies with the seasonal progression. The ERL-D report indicates that the food availability in the first few days after feeding begins is also a factor. The report of ANSP suggests that some other food (hay infusion is suggested) be used during this critical period in future testing.

Each of the above suggestions have merit. However, the nonstandard nature of each of the suggested remedies makes recommending such additions to the guidelines difficult. In this author's opinion, a test with the desired species with all factors except the toxicant initially comparing weights of the usual 5 duplicates as an operations check should be conducted. The necessary changes to reach a minimum mean weight of no less than 0.1 g per animal at 28 day post hatch could then be made. This would allow comparison of the expected results throughout the system and a thorough familiarity with the system before any testing is begun. Any effect of variations in flow, lighting, tank position, etc., could also be controlled. A similar pretest check on the toxicant additions and chemical analysis system should also be conducted but for less time (a week is suggested). These additions to the guidelines would save time and funds. If this is not done, at least two tests on each chemical should be conducted, a minimum weight for the controls should be set, and provision should be made in the initial planning for retesting of any material in which this minimum is not met. Another check parameter that could be included in the guidelines is to discard any test

where surviving animals in partial kill concentrations are larger than the controls, indicating that food and/or space stress was a problem. (The three laboratories adjudged satisfactory met this criteria as there was no difference in fish mean weight in chambers with fewer fish.)

The condition that must be worked toward in any continuous and continuing testing procedure is uniformity in all parameters except that being tested.

These guidelines in general meet this need. The only part of the guidelines that appear to be in need of modification is the type and/or quality of food used.

Summary of recommendation for use of these guidelines in toxicity evaluation.

1. Conduct a training session for the principal biologist prior to start up of any system following the guidelines.
2. Require all testing laboratories to conduct a full scale test without toxicant to evaluate their laboratory techniques.
3. Produce a set of forms for daily recording and post test compilation including statistics (should include number of chemistries and other information user needs).
4. Assign chemicals with similar solubilities, volatilities, and other physical properties to each test program as much as possible to increase productivity of test systems.
5. In lieu of requirement on #2 above, set a minimum control mean weight of 0.1 gr per fish. (This requirement is not as useful as #1 because chemicals will be lost and repeats will be required while requirement two will preclude any laboratory doing the work if they have unsatisfactory techniques.)

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Appendix 1

Participating Organizations

ABI	Applied Biology, Inc.	Mark Smith
ANSP	Academy of Natural Sciences of Philadelphia	Dr. Arthur Scheier
ERCO	Energy Resources Co.	Timothy Ward
ERL-D	Environmental Research Laboratory-Duluth	Armond Lemke
JEI	Jones Edmunds, Inc.	Mary Leslie
MBL	Marine Bioassay Laboratory	John Hansen
WFTS	Western Fish Toxicity Station	Dr. Alan Nebeker

Guidelines for Conducting Flow-Through Early Life Stage Toxicity Tests with Fathead Minnows for use in the USEPA, OTS-ORD Round Robin Test

1. In an Early Life Stage Toxicity Test with fathead minnows, organisms are exposed to toxicant during part of the embryonic stage, all of the larval stage and part of the juvenile stage. The organisms are examined for statistically significant reductions in survival and weight in order to determine lower and upper chronic endpoints.

   A lower chronic endpoint is the highest tested concentration (a) in an acceptable chronic test, (b) which did not cause the occurrence (which was statistically significantly different from the control at the 95% level) of any specified adverse effect, and (c) below which no tested concentration caused such an occurrence.

   An upper chronic endpoint is the lowest tested concentration (a) in an acceptable chronic test, (b) which caused the occurrence (which was statistically significantly different from the control at the 95% level) of any specified adverse effect and (c) above which all tested concentrations caused such an occurrence.

2. Not enough information is currently available concerning early life stage tests with fathead minnows to allow precise specification of details for all aspects of the test. Enough such tests have been conducted and enough aspects have been studied, however, to indicate that these Guidelines are appropriate. A prudent course of action for anyone planning to conduct such tests would be to initially conduct a test with no toxicant to gain experience and to determine if the requirements of sections 10, 11, 19, 20, 26 and 27 can be met using the

planned water, food, procedures, etc. If a solvent may be used in the preparation of a stock solution, it would also be prudent to test one or more concentrations of one or more solvents at the same time (see Section 4). General information on such things as apparatus, dilution water, toxicant, randomization of test chambers and organisms, and methods for chemical analyses, can be found in Draft #10 of the proposed ASTM Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.

3. Tests should be conducted with at least five toxicant concentrations in a geometric series and at least one control treatment. The concentraiton of toxicant in each treatment, except for highest concentration and the control treatment, should usually be 50 percent of that in the next higher one.

4. If a solvent other than water is used to prepare test solutions, a solvent control (at the highest solvent concentration present in any other treatment) using twice as many test organisms and test chambers as the other treatments is required in addition to the regular control, unless such a control has already been tested in the same water with the same species of fish, food, and test procedure and the water quality has not changed significantly. A concentration of solvent is acceptable only if it is (or has been) shown that that concentration or a higher one does not cause an increase or decrease in survival or weight at the end of the test that is statistically significantly different from the control at the 95% level using a two-tailed test.

5. For each treatment (toxicant concentration and control) there must be at least two replicate test chambers each containing one or more embryo

cups with at least 60 embryos divided equally between the embryo cups at the beginning of the test.

6. Two test chambers have been used routinely:

   a. Twenty fish have been tested in a chamber which is 16 cm x 44 cm x 18 cm high with a 16 cm x 18 cm 40-mesh stainless steel screen 6 cm from one end, with a water depth of 12.8 cm and with a flow rate of 190 ml/minute.

   b. Fifteen fish have been tested in a chamber which is 6.5 cm x 18.0 cm x 9.0 xm high with a 6.5 cm x 9.0 cm 40-mesh stainless steel screen 2.5 cm from one end, with a water depth of 4.5 cm and with a flow rate of 15 ml/minute.

   All of the above are inside dimensions. In both test chambers the water depth is controlled by a standpipe located in the smaller screened compartment with the test solution entering at the other end of the test chamber.

7. Embryo cups should be glass cylinders about 4.5 cm inside diameter and about 7 cm high with 40-mesh nylon or stainless steel screen glued to the bottom. The embryo cups must be suspended in the test chamber in such a way as to insure that the organisms are always submerged and that test solution regularly flows into and out of the cup without agitating the organisms too vigorously. A rocker arm apparatus driven by a 2 r.p.m. motor and having a vertical-travel distance of 2.5 - 4.0 cm has been successfully used, as have self-starting siphons that cause the level of solution in the test chamber to rise and fall.

8. Any water in which fathead minnows will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for early life stage toxicity tests with fathead minnows.

9. A 16-hr light and 8-hr dark photoperiod should be provided. A 15- to 30-minute transition period at "lights on" and "lights off" may be desirable. Light intensities from 10 to 100 lumens at the water surface have been used successfully, but the intensity should be about the same for all test chambers. Light should be provided by wide-spectrum (color Rendering Index > 90) fluorescent lamps.

10. Tests should be conducted at 25°C. The temperature in each test chamber should be between 24 and 26°C at all times and must be between 20 and 28°C at all times. If the water is heated, precautions should be taken to assure that supersaturation of dissolved gases is avoided and total dissolved gases should be measured at least once during the test in the water entering the control treatment.

11. The dissolved oxygen concentration should be between 75 percent and 100 percent saturation at all times in all test chambers. At no time during the test should one test chamber have a dissolved oxygen concentration that is more than 1.1 times the dissolved oxygen concentration occurring in another chamber at the same time.

12. The flow rate of test solution through the test chambers must be great enough to maintain the dissolved oxygen concentration (see sections 11 and 22) and to insure that the toxicant concentrations are not decreased significantly due to uptake by test organisms and material on the sides and bottoms of the chambers.

13. A test begins when embryos in embryo cups are placed in test solution and ends 32 days later.

14. Embryos and fish should not be treated to cure or prevent disease or fungus before or during a test.

15. Embryos should be obtained from a fathead minnow stock culture maintained at 25°C and a dissolved oxygen concentration between 75% and 100% saturation with a 16-hr light and 8-hr dark photoperiod. Frozen adult brine shrimp has been successfully used as a food for adult fathead minnows. The maximum production of embryos by fathead minnows has been obtained in a 30 cm x 60 cm x 30 cm deep chamber with a water depth of 15 cm when 15 cm x 30 cm quadrants are formed with stainless steel screen and one male, one female and one or two substrates are placed in each quadrant. Half-round spawning substrates (Benoit and Carlson, 1977) with an inside diameter of 7.5 cm and a length of 7.5 cm have been used successfully.

16. The afternoon before a test is to begin, all of the substrates should be removed from an appropriate number of tanks in the stock culture unit and should be replaced about the time the lights are turned on the next morning. Enough (at least three) substrates with embryos on them should be removed six hours later and soaked in dilution water for two hours. For each individual substrate the embryos should be gently separated (Gast and Brungs, 1973) and removed and visually examined using a dissecting scope or a magnifying viewer. Empty shells and undeveloped and opaque embryos should be discarded. If less than 50 percent of the embryos from a substrate appear to be healthy and fertile, all the

embryos from that substrate should be discarded. Single embryos with no fungus or partial shells attached are preferable, although embryos with partial shells attached and clumps of two or three embryos (with or without separation) have been used successfully. An approximately equal number of acceptable embryos from one substrate should be impartially distributed to each embryo cup and the process repeated for at least two more substrates until the proper number of embryos have been placed in each cup to give at least 60 embryos per treatment. The embryo cups should be standing in dilution water when the embryos are being distributed and then the cups should be randomly placed in the test chambers.

17. Twenty to 24 hours after they are placed in the embryo cups, the embryos should be visually examined under a dissecting scope or magnifying viewer and all dead embryos should be counted and discarded. Embryos that are alive but heavily fungused should also be counted and discarded. Forty to 48 hours after the start of the exposure all dead and heavily fungused embryos should be counted and removed. The remaining healthy, fertile embryos should be impartially reduced to the desired number of test organisms (at least 30 per treatment). If more than about 35 percent of the embryos in the control treatment are discarded within the first 48 hours of the test because they are dead or heavily fungused, it will probably be cost-effective to restart the test. In addition, if toxicant related effects are seen at 48 hours, it will probably be cost effective to restart the test since all of the toxicant concentrations will probably cause adverse effects. Each day thereafter dead embryos should be counted and discarded.

18. In each treatment, when hatching is about 90% completed or 48 hr after first hatch in that treatment, the live young fish should be counted and the live fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal in either swimming behavior or physical appearance should be counted. All of the normal and abnormal live fish should be released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The range of time-to-hatch (to the nearest day) in each cup should be recorded.

19. A test should be terminated if the average percent of embryos (based on the number of embryos after thinning) that produce live fry for release into test chambers in any control treatment is less than 50 percent or if the percent hatch in any control embryo cup is more than 1.6 times that in another control embryo cup.

20. The flow rate, size of the test chamber and the amount of food added should be such that the average weight of the control fish at the end of the test would not be significantly greater if only half as many fish were tested per test chamber.

21. Each test chamber containing live fish over two days old must be fed live newly hatched brine shrimp at least two times a day at least 6 hrs apart (or three times a day about four hours apart) on days 2-5 after hatch and at least five days a week thereafter. They must be fed at least once a day on all other days. Other food may also be provided in addition to the above. The amount of food provided to each chamber may be proportional to the number and size of fish in the chamber, but each

chamber must be treated in a comparable manner. Quantifying the amount of live newly hatched brine shrimp to be fed is difficult, but the fish should not be excessively overfed or underfed. A large buildup of food on the bottom of the chamber is a sign of excessive overfeeding. A sign of not feeding enough of the right kind of food is that in the sideview the abdomen does not protrude.

22. Test chambers should be cleaned often enough to maintain the dissolved oxygen concentration (see sections 11 and 12) and to insure that the toxicant concentrations are not decreased significantly due to sorption by matter on the bottom and sides. In most tests if the organisms are not overfed too much and the flow rate is not too low, removing debris from the bottom once or twice a week should be adequate. With some toxicants that promote growth of bacteria the sides and bottoms should be cleaned more often. Debris can be removed with a large pipette and rubber bulb or by siphoning into a white bucket. A dark tip on the pipette or siphon should help fish avoid being sucked up, but the pipette or bucket should be examined to insure that no live fish is discarded.

23. Temperatures should be recorded in all test chambers once at the beginning of the test and once near the middle of the test. In addition, temperature should be recorded at least hourly in one test chamber throughout the test. The dissolved oxygen concentration should be measured in each treatment at least once a week during the test. Hardness, pH, alkalinity, and acidity should be measured once a week in the control treatment and once in the highest toxicant concentration.

The concentration of toxicant should be measured at least twice a week in each treatment.

24. Dead fish should be removed and recorded when observed. At a minimum 11, 18, 25 and 32 days after the beginning of the test, the live fish should be counted and the fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic and grossly abnormal in either swimming behavior or physical appearance should be counted.

25. The fish should not be fed for the last 24 hours prior to termination on day 32. At termination the weight (wet, blotted dry) of each fish that was alive at the end of the test should be determined. If the fish exposed to toxicant appear to be edematous compared to control fish, determination of dry, rather than wet, weight is desirable.

26. A test is not acceptable if the average survival of the controls at the end of the test is less than 80 percent or if survival in any control chamber is less than 70 percent.

27. A test is not acceptable if the relative standard deviation (RSD = 100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

28. Data to be statistically analyzed are:
    (A) percent of healthy, fertile embryos at 40-48 hours
    (B) percent of embryos that produce live fry for release into test chambers

(C) percent of embryos that produce live, normal fry for release into test chambers

(D) percent of embryos that produce live fish at end of test

(E) percent of embryos that produce live, normal fish at end of test

(F) weights of individual fish that were alive at end of test.

Although item A is based on the number of embryos initially placed in embryo cups, items B, C, D, and E are based on the number of embryos after thinning.

29. Dichotomous data (live-dead, normal-abnormal) should be analyzed using contingency tables (Sokal and Rohlf, Biometry, 1969, p. 587) or log linear techniques. For weight data the individual fish are used as the replicates unless a two-tailed F test indicates that differences between replicate test chambers are not negligible. Weight data may be analyzed using Bartlett's test and one-way analysis of variance, but Dunnett's procedure (Steel and Torrie, Principles and Procedures of Statistics, 1960, p. 111) should be used to identify treatments producing weights that are statistically significantly lower than those of the controls at the 95% level.

30. Although the results of the analyses of all six types of data in section 23 should be reported, the lower and upper chronic endpoints are only based on statistically significant reductions in survival and weight at the end of the test (items D and F). Item A is apparently relatively insensitive and item B is included in item D. In addition, abnormal fish seem to weigh less than normal fish and so will be covered in item F. Also, since the distinction between normal and abnormal is subjective, this kind of data is expected to be less reproducible from

one investigator to another than the other kinds of data. Although items D and F are considered primary, the other items are included because they may provide useful information.

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